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Soluble Sulfides in Rice Fields and Their in Vitro Effects on Rice Seedlings.

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ABSTRACT

A silver sulfide electrode was used for measurement of soil sulfide levels in Louisiana rice fields. The observed potential obeyed the Nernst equation as a function of sulfide ion activity ($^{a}S^{=}$) or concentration $[S^{=}]$ and the calibration curve could be extended to $[S^{=}] = 10^{-20}$ M by direct calculation from this equation, on the single assumption that complexing of $S^{=}$ with protons produced a sum of concentrations $[S^{=}]$, $[HS^{-}]$, and $[H_2S] \geqslant 10^{-7}$ M. Thus free $[S^{=}]$ was determined by its simultaneous measurement with pH. Also, sulfide ion concentrations could be determined by direct potentiometry or potentiometric titration. The response rate of such electrodes suggests that continuous monitoring of some changing systems is feasible. The results were reproducible and highly correlated (r = .9965) with the standard methylene blue method for determination of hydrogen sulfide.

Hydrogen sulfide levels ranged from .00005 to .64128 ppm in Louisiana rice fields during the tillering and ripening stages of rice plant development. A peak of H_2S accumulation coincided with the highly reduced conditions occurring at the heading-flowering stage of the rice plant. H_2S levels prevalent in rice fields during the heading-flowering stage were toxic to rice plants in vitro and significantly higher than those predicted from chemical equilibrium theory. Field measurements of soluble sulfides were not made at any other period during the rice growing season.

The two most important factors regulating H_2S accumulation in Louisiana rice fields were oxidizable carbon and soil pH.

The hypothesis that H_2S is removed from the soil solution by the soil clay fraction was supported. Bentonite 2:1 type clay, a major constituent of some rice soils, showed $S^{=}$ sorption in the range of 2.68-2.82 ppm/g; this was due to a combination of physical adsorption and chemical fixation.

Rice seedling biological assays showed significant inhibition of the respiration of rice roots pretreated with H_2S at levels within the range of those measured in Louisiana rice fields. Drastic inhibition of the activities of catalase, peroxidase and ascorbic acid oxidase in roots of H_2S-pretreated rice seedlings was also shown.

These data provide empirical links -- reduction of rice root oxidative capacity and other physiological functions such as nutrient uptake -- between hydrogen sulfide levels occurring in Louisiana rice fields and toxicant diseases manifested as reduced grain yields.

INTRODUCTION

Submerging a soil under water instantly sets in motion a series of physical, microbial, and chemical processes. These processes include retardation of gaseous exchange between soil and air as well as a sequence of electrochemical and chemical changes which bring about biochemical reduction and accompany the exhaustion of oxygen in the soil. These changes result in anaerobiosis and the accumulation of toxic substances in submerged soils (55, 56).

Anaerobic metabolism of bacteria in submerged rice soils can produce an array of organic substances whose composition, concentration, and stability are governed by the nature and content of organic matter, environmental factors, microbial activity, and duration of submergence (1, 74, 75, 82). One of the toxic metabolites produced by anaerobic bacteria is hydrogen sulfide (H_2S). Under the anaerobic conditions prevailing in rice fields, attention has been focused principally on ferrous iron (Fe^{++}) and the soluble sulfide species--H_2S, HS^-, $S^=$ -- and their possible toxicity to rice roots (50, 78).

The possible presence of hydrogen sulfide in rice soils raises the question of how its known toxicity as an enzyme inhibitor might influence events in a rice field (6, 24, 41, 55, 64, 70). The formation of free H_2S in soil has been demonstrated conclusively and linked to the so-called Akiochi (Autumn decline) disease of rice in Japan (72, 73). Postulated consequences of the possible presence of this gas in rice fields have been proposed with respect to the Mentek disease of rice in Java and the Straighthead disease in the USA (5, 6, 72, 73).

Furthermore, symptomless toxicant disorders (diseases) of rice, which exert an effect on grain yield have been postulated to be caused by H_2S (24).

Concentrations of total combined and free sulfides from Louisiana soil samples range up to 45 ppm (15, 24, 63, 70). Combined sulfides, principally insoluble FeS, are in equilibrium with the soluble sulfide species (44, 45, 55, 56). Predictions from the chemical equilibrium theory (24) of soluble sulfide concentrations within the range of levels known to cause toxic effects to rice seedlings ($\geqslant$ 0.07 ppm) (6, 24, 41, 43, 56), combined with soluble sulfide measurements of field samples by the colorimetric (methylene blue) method (15, 24, 25, 64) have given rise to the view (24, 55) that soluble sulfide levels may cause toxicant disease in Louisiana rice fields. Consequently, work was done to further test the hypothesis that hydrogen sulfide is a yield-reducing factor in Gulf coast (USA) rice fields.

LITERATURE REVIEW

I. Some Characteristics of Submerged Soils

1. Soil reduction:

After submergence, an arable soil rapidly changes from an oxidized to a reduced state due to the consumption of oxygen and the accumulation of reduced substances in the soil. Theoretically, soil reduction proceeds in a sequence predicted by thermodynamic analysis and produces an array of redox systems ranging from the O_2-H_2O system at submergence to the H^+-H_2 system in strongly reduced soils (52, 55, 56, 83). Experimental evidence of this sequential reduction can be found in a number of papers presented in recent review articles concerned with soils (4, 47, 51, 52, 56, 62, 81).

Reduction of a submerged soil is caused by the anaerobic metabolism of soil microorganisms. It proceeds in the following sequence: nitrate, manganese dioxide, ferric hydroxide and intermediate breakdown products of organic matter, sulfate, and perhaps, phosphate (52, 55, 56). Furthermore, reduction affects the soil electrochemical characteristics. These electrochemical changes accompanying the reduction of a soil are a decrease in redox-potential (4, 14, 51, 56, 70), an increase in pH (55, 62, 70), and an increase in specific conductance (56, 65). Thus, soil reduction affects both the soil physiochemical characteristics and consequently the inorganic and organic reduction products.

The question of the effects of toxic products versus reduction per se on rice plants was recently tested without conclusive results (43, 56). This question is important because of the extensive evidence linking the root oxidizing power of rice varieties with resistance to physiological disorders. Soil reduction does not appear detrimental to rice growth except at potentials low enough for accumulation of reduction products (14, 15, 34, 41, 43, 56, 83). Also, the root of the rice plant, which is generally accepted as predominantly oxidative, was not oxidative at its flowering stage of growth (7, 8, 41), at a time in which reduction products may be accumulated in the soil and injure the plant.

Soil science literature reflects an overemphasis of the regulatory role of iron in retarding soil reduction and detoxifying H_2S (34, 39, 51, 52, 74, 75). Motomura (47) showed that only one form of ferrous iron produces exchangeable cations in submerged soils. Four other forms were exchangeably adsorbed, either strongly or in the fixed state, on soil colloids and/or organic compounds according to the kinds of clay minerals and/or exchangeable sites (47, 55, 82, 83). Also, it is unlikely that $Fe(OH)_3$ exerts any appreciable restraint on the reduction of a soil, despite its existence in large quantities in the soil. This is due to the low solubility of $Fe(OH)_3$ and the absence of organisms that can reduce $Fe(OH)_3$ by intrinsically biological pathways (11, 65)

The redox-potential is a measure of the intensity of soil reduction. It reveals nothing about the concentrations of reduction products. Soils at nearly the same redox-potential may have different concentrations of reduction products; conversely, soil having nearly

the same concentrations of reduction products may have different redox-potentials (56, 83). Yet, ecologically it is the concentration of soluble reduction products that matters (24, 57), for rice roots have only a limited capacity for oxidizing and rendering innocuous the reduction products in their milieu (41, 46).

The change in pH value of the soil after submergence has an important bearing on reduction products and the nutrition of the rice plant (24, 55, 56). Iron deficiency in alkaline soils and toxicity in Latosolic soils is supposedly due to this change in soil pH (47, 57, 81). Also, the concentrations of HS^- produced from H_2S is a function of soil pH. HS^- concentration increases from 0.2 % at pH 4.5 to 58.0 % at pH 7.0. Theoretical values of H_2S were calculated under Louisiana rice field conditions and showed concentrations of 100, 16.7, 1.7, 0.17 and 0.017 ppm at pH values of 5.0, 5.5, 6.0, 6.5 and 7.0, respectively (24). Levels of H_2S have been detected in Louisiana rice fields by indirect methods of measurement which exceed toxic concentrations reported in the literature at naturally-occurring pH levels of 5.8-6.8 (14, 15, 24, 70).

Recent studies (58, 59) have made use of Eh-pH stability diagrams as a diagnostic tool in differentiating suitable soils for rice culture. The basis of the Eh-pH system are not sound due to the following factors: potentials of submerged soils are not static, redox-potential systems of submerged soils have not been clearly defined, and high negative potentials cannot be quantitatively explained by the iron system alone. Also, the theoretical conversion factor may vary from -30 to -180 mV per pH unit at 30 C for the soil system, although many scientists uncritically use -60 mV/pH (34, 57, 62). Furthermore,

theoretically, a tenfold increase in the concentration of a reduction product in the soil solution can lower the potential for many soil redox-systems by only -60mV, a figure considerably less than random variation in some submerged soils (34, 62, 70).

2. Sulfate reduction and accumulation of sulfides:

Reduction of sulfate to sulfide takes place in a submerged soil after it has undergone appreciable reduction. A complete review of the ecology and physiology of sulfate-reducers can be found elsewhere (1, 3, 60, 69, 84).

Although many species and genera of sulfate-reducing organisms are present in the soil, only bacteria of the genus Desulfovibrio, Beijerinck, 1895 (Kluyver and Van Neil, 1936), are predominant in reducing soil sulfate (3). They are strict anaerobes and function best at low redox-potentials and near neutral pH (60, 69, 84). Members of Desulfovibrio are capable of reducing sulfate, sulfite, thiosulfate, and elemental sulfur to sulfides (3). Other genera of bacteria capable of reducing sulfate are Clostridium (Prazmowski, 1880) and Bacillus (Cohn, 1872), but only under certain conditions (3, 11).

The reduction of sulfate is not the only means by which sulfide is produced by microorganisms. H_2S is produced by many bacteria as a product of putrefaction (9, 10). The decomposition of organic sulfur-containing compounds leads to the production of sulfides in submerged soils (3, 73). Under most soil conditions, however, these mechanisms of sulfide formation are minor factors as compared to the reduction of indigenous soil sulfate or that applied to the soil (10, 84).

The reduction of sulfate is especially important in rice soils, because it is a common practice to submerge rice soils and to add

considerable amounts of sulfate fertilizers to rice soils each year (55, 70). Such conditions in rice culture bring about anaerobiosis and reduction of sulfate. It is a common view that accumulation of soluble reduction products may exceed levels toxic to the rice plant but proof has been lacking. Takijima and Mitsuhiro (77) measured the accumulation of total soil sulfides as a function of time during the rice growing season. They reported two distinct peaks, one at the beginning and the other at the end of the summer season. Yamane and Sato (82) found a sharp rise of total sulfide production in a muck soil within 1 to 3 weeks after submergence. In laboratory experiments, total sulfide accumulation increased with time. The rate of increase was positively related to the organic matter content of a given soil (49).

Total sulfide levels of submerged soils, reflected mainly as iron sulfide, may increase to concentrations in excess of 150 ppm (3), and levels as high as 2000 ppm have been reported (23). In Louisiana rice fields, concentrations of total sulfides increased from 0.2 ppm within 5 to 7 days after submergence to levels varying from 10 to 45 ppm at the end of the season (63, 64). Sturgis (70) reported a total sulfide content of 29.9 ppm in Sharkey clay loam 3 weeks after submergence. By contrast, recent studies have produced values of free soluble H_2S ranging from 0 to 1.6 ppm in Crowley silt loam and Hockley fine sandy loam soil samples (14, 15, 27). Analyses of Louisiana soil samples revealed apparent soil solution concentrations of H_2S on the order of 4, 8 and 12 ppm in certain sites (24, 25, 26, 27).

The occurrence of such relatively high concentrations of free, soluble H_2S in the presence of free ferrous iron in Louisiana rice

soils (24, 25, 51, 70) and the absence of characteristic odor both suggest that hydrogen sulfide is not free in the soil solution (24) but is under a dynamic physiochemical equilibrium with respect to soil conditions (47) and the specific nature of the soil clay fraction (36).

There are many reported variables influencing sulfide reactions in submerged soils (24, 57, 74, 75). For example, it is known that the soluble portion of FeS is not completely ionized (57), that iron-sulfide species in the soil are a function of the ratio of concentrations between H_2S and metal salt (24), and that H_2S may be adsorbed by the soil clay fraction (36). The results of numerous experiments showed the vigorous evolution of gases from the furrow slice layer in the peaty rice soils of Japan. However, the free H_2S content of these gases was so slight that it was disregarded as an inhibitor of plant growth (77).

Reports of further inconsistencies with respect to H_2S determinations have come from iron additions to muck soils, which had little effect in preventing the evolution of H_2S from these soils (82). Bloomfield (9) concluded that although soluble ferrous iron was present in the soil, as much as half the sulfate in the soil was sometimes lost as H_2S in laboratory experiments. In another study he found that sulfate disappeared almost completely within 10 days after submergence. About 40 % of this sulfate was lost as H_2S in a linear fashion with time over a pH range of 5.8-8.3, despite the presence of nearly threefold excess of Fe^{++} in his soil samples (10).

Traces of H_2S evolved in laboratory and field experiments have been generally overlooked in relation to physiological disorders of the rice plant. In laboratory experiments, Connell and Patrick (15)

showed that "little sulfide" (7 ppm) existed as H_2S in some Louisiana rice soils if excess ferrous iron was present. They demonstrated the indigenous presence of 2 ppm H_2S in Hockley sandy loam soils. Hollis (24) found that theoretical values of H_2S exceeded the levels detected by indirect measurements in Louisiana and are in the range of levels toxic to rice plants in vitro. He based his calculations on measured values of Fe^{++}, Eh and pH in Louisiana rice fields.

As the above mentioned levels are within the range of theoretically-predictable concentrations of H_2S and occur in the presence of normal ferrous iron concentrations in Louisiana fields (51, 70), rice should exhibit toxicity symptoms, and H_2S should be detectable by odor. A comparable set of these circumstances appears to exist in the major rice producing areas of the world (6, 56, 72, 73). However, there has been little or no expression of concern in the literature over these inconsistencies. This may be due to the feeling that such low H_2S values may have resulted from error or that the in vitro toxicity test results are not directly applicable to the rice field environment (24). More important is the fact that there have been no reports of in situ measurements for concentrations of naturally produced H_2S in submerged soils. Also, conventional methods of determination tend to show higher values of H_2S. This may be due to the conditions of the assay which tend to release adsorbed H_2S.

II. Sulfide toxicity and rice disorders

The presence of sulfides in submerged soils raises the problem of H_2S toxicity. Mitsui and his associates (41, 42, 43, 44, 45) have demonstrated the injurious effects of H_2S on rice plants. Exposure of

rice roots to a solution containing 0.07 ppm H_2S was sufficient to cause wilting of 42 % of the leaves at the end of 200 hr. At an H_2S concentration of 2 ppm the same degree of wilting was observed at the end of 48 hr. Thus, it appears that injury to rice roots by H_2S is a function of H_2S concentration in the soil solution and the period of time the roots are in contact with it. Concentrations as low as 0.1 ppm of H_2S in culture solutions were toxic to rice plants (57).

H_2S is a well known inhibitor of the enzymes of aerobic respiration (18, 29, 32, 33, 35), and iron-containing enzymes also have been shown to be inhibited by H_2S (48, 53, 54). Furthermore, H_2S has been considered one of the principal inhibitors of nutrient uptake by rice plants. Mitsui (43) showed evidence linking phosphorus uptake with oxidative phosphorylation, potassium accumulation with nucleic acid metabolism, and nitrogen absorption with the TCA cycle.

In the light of these laboratory studies and the principles underlying sulfate reduction in submerged soils, it is interesting to consider the cause or causal agents and etiology of certain physiological disorders of lowland rice. Recent reviews of literature indicated that either ferrous iron or some form of sulfide is suspected as a cause of several physiological disorders of rice around the world (6, 24, 41, 50, 56, 72, 73). These disorders or so-called diseases include Bronzing in Ceylon (72, 73, 78, 80), Akagare and Akiochi in Japan (72), Straighthead disease in the U.S.A. (5, 79) and Japan (57, 73), Amyitpo in Burma (72) and Suffocation disease in Taiwan (73).

"Akiochi" disease or autumn decline, now known to exist in at least two principal soil types, is responsible for reduced yields on more than 20 % of the total area planted with rice in Japan (24, 72).

Akiochi on degraded paddy soils ("Akiochi-DPS") is related to sulfide residues on root surfaces and/or very small quantities of H_2S which are not detectable by odor. Its actual cause, however, remains obscure (76). Akagare disease is promoted by the application of organic matter to the soil or production of H_2S in the soil (6).

Tanaka, et al. (78), concluded that if concentrations of both ferrous iron and sulfide in the soil solution are higher than certain critical levels, the oxidizing power of rice roots is destroyed by sulfide and the rice plants become more vulnerable to iron toxicity (6, 50). They found that bronzing symptoms developed at 100 ppm Fe^{++} and 7 ppm $S^{=}$ or 300 ppm Fe^{++} and 3 ppm $S^{=}$ (78).

Straighthead disease is characterized by erect, unbending panicles due to failure of grain development, accompanied by distortion of the glumes and bracts (79). The disease occurs most commonly under continuous flooding on sandy soils of low clay content and abundant, decomposable organic matter (5). In Texas, Straighthead disease severity exhibits continuous variability on Hockley fine sandy loam (24). Patrick and Connell (15) reported 2 ppm soluble sulfide indigenous to this particular soil, which could have some toxic effect on rice plants. On old cotton land, arsenic is a cause of Straighthead, but the more general cause is unknown (5, 24, 79).

Hollis (24) is of the opinion that symptomless diseases occur in Gulf Coast rice areas. These diseases are not detectable until late season effects on rice plants appear. These effects include nutrient deficiencies, fungus diseases associated with weakened plants, or simply differences in grain yields. They are truly symptomless in a specific sense if the only observable differences are in grain yield.

MATERIALS AND METHODS

The experiments reported herein fell into two broad groups. The first group included evaluation of the specific silver-sulfide membrane electrode (sulfide electrode) for measurement of the soluble sulfide levels in submerged soils, for measuring sorption of sulfide by clay in response to sulfide concentrations, and for the testing of these observed sulfide levels against chemical theory. In the second group of experiments, a seedling biological assay was used to measure certain respiratory and enzymatic responses of rice root tissues to H_2S levels equivalent to field sulfide levels.

Electrodes and apparatus:

A model 94-16 silver-sulfide membrane electrode and a double-junction reference electrode model 90-02 were obtained for this study from Orion Research, Inc., Cambridge, Mass. The operating characteristics of the sulfide electrode have been described previously (12, 16, 28, 61, 66, 71) and are summarized in Table 1.

Electromotive force (e.m.f.) was determined with an Orion model 407 ionanalyzer obtained from Orion Research, Inc., Cambridge, Mass. Measurements of pH were made with a Beckman Zeromatic pH meter, using a Coleman tripurpose shielded glass electrode associated with a reference electrode.

Table 1. Characteristics of the silver-sulfide membrane electrode.

Concentration range	10^{-0} - 10^{-7} total silver or sulfide. 10^{-0} - 10^{-25} free silver or sulfide.
Temperature range	1 - 100 C
pH range	0 - 14
Slope at 25 C	29.6 mv/sulfide/decade 59.2 mv/silver/decade
Transient response time	5 msec. - 2 min.
Interferences with anions	Sulfide ion: none. Silver ion: Hg^{++} (K:AgHg = .08)
Temperature coefficient	+0.05 mv/C in 0.1M Na_2S, or in 1.0M Na_2S. -0.40 mv/C in 0.1 M $AgNO_3$
Resistance	0.5-1 megohm.

Calibration curves and sulfide measurements:

The potential E (mV) of the sulfide electrode was measured in a serial dilution of sodium sulfide solutions made with 1.0 M sodium hydroxide (NaOH) to maintain a constant ionic strength (μ= 1.0). Standard solutions of sulfide were protected from oxidation and carbonate formation by purging with N_2 both during and after preparation. Stock solutions of 0.1 M, or 0.01 M in silver concentrations were prepared by direct weighing of $AgNO_3$ (M.W. = 176.87). All solutions were stored in glass containers at room temperature (24 $\pm$ 2 C). Silver nitrate solutions were protected against direct light. All chemicals were of reagent grade.

Titrations were made by using silver nitrate solutions with the sulfide electrode as an end-point detector by plotting the potential in (mV) against ml of silver nitrate. An extended calibration curve of experimental sulfide values was obtained by plotting the sulfide electrode potential E (mV) developed versus the sulfide ion activities ($^aS^=$) or concentrations [$S^=$] on semi-logarithmic paper. The sulfide electrode response to H^+ ion activity (pH) was tested in a known sulfide solution (29.5 ppm $S^=$) while varying the pH by HCl or borate buffer (28). Molarity of sulfide solutions was calculated from titration curves by equation 1;

$$M_s = \frac{V_t \times M_t}{2\ V_s} \quad \text{. . . . . (Eq. 1)}$$

where:

M_s = concentration of sulfide ion in the solution.

V_t = volume of $AgNO_3$ titrant added.

M_t = concentration of $AgNO_3$ titrant added.

V_s = volume of unknown solution.

To convert from moles per liter of sulfide ion to parts per million or mg/ml, the M_s values were multiplied by a conversion factor of 32,064.

Comparison of sulfide electrode measurements by the methylene blue method:

Sulfide concentrations of standard Na_2S solutions or H_2S solutions were determined by both the sulfide electrode and the widely used, conventional methylene blue method (67). The electrode method is relatively new, but quite sensitive and has the advantage of being applicable in the field (19, 28, 71).

Sulfide levels in rice fields

Measurements of soluble sulfides were made directly in rice fields late in the 1970 growing season (during July and August) and in the laboratory on soil samples removed from fields in half-pint, sealed jars. Free sulfide $^aS^=$ was determined for each sample. This was accomplished by recording the potential developed by the sulfide electrode simultaneously with the sample pH. Electrode potentials were recorded after no apparent increase in potential was observed. Measured potential values were converted to $pS^=$ levels by the use of the extended calibration curve. These $pS^=$ levels were used in equation 2, according to Garrels and Christ (21), to determine p $[H_2S]$ and then H_2S concentrations.

$$p[H_2S] = 2\ pH + pS^= - 20.9 \quad \text{(Eq. 2)}$$

Site pH and Eh values were recorded also. All Eh values were corrected to pH 7.0 by adding (7.0 - pH observed) (-60 mV) to the observed Eh

value and corrected for the saturated calomel electrode with the following equation:

$$Eh_7 = 241 + Eh \text{ observed} + (7\text{-pH observed}) (-60mV) \ldots \quad \text{(Eq. 3)}$$

A nested analysis of variance was conducted for H_2S, pH and Eh levels where sites were nested within fields within weeks (13).

Comparison of measured H_2S levels by the theoretically calculated values:

Theoretical H_2S values were calculated on the basis of an experimental range of Eh values (Eh = -0.059 to -0.118V) and of ferrous iron values (pFe^{++} = 3 to 4) in Louisiana rice soils (25, 26, 51, 63, 70). The use of these variables, in addition to soil pH, in the Nernst equation produced equation 4 (21).

$$Eh = 0.14 - 0.177\ pH + 0.059\ pFe^{++} + 0.089\ pH_2S \ldots \quad \text{(Eq. 4)}$$

The derivation of equation 4 produces theoretical limits of H_2S as shown in equations 5 and 6 (21).

$$pH_2S = 2\ pH - 2.81 \ldots \quad \text{(Eq. 5)}$$

$$pH_2S = 2\ pH - 4.40 \ldots \quad \text{(Eq. 6)}$$

These equations yield a minimum limit of (Eh = -0.059 V and pFe^{++} = 3.0) and a maximum limit of (Eh = -0.118 and pFe^{++} = 4.0), respectively. The Chi-square test (13) was used for statistical comparisons of the experimental and theoretical data.

Regression of H_2S on Fe^{++}, Mn^{++}, oxidizable carbon, clay percent, Eh_7 and pH:

The relationship of variation in H_2S levels, in samples collected in rice fields during August, to variation in soil characteristics was evaluated by multiple regression analysis (13).

Characteristics chosen to reflect various aspects of rice field sulfide levels were as follows:

Y: H_2S level

X_1: Ferrous iron concentration (Fe^{++} ppm)

X_2: Percentage of oxidizable carbon (O.C%)

X_3: Percent of clay fraction (C%)

X_4: Sample hydrogen ion concentration (pH)

X_5: Sample corrected redox-potential (Eh_7)

X_6: Manganous (Mn^{++} ppm)

The following model for H_2S concentration was used:

$$Y = \alpha + b_1X_1 + b_2X_2 + b_3X_3 + b_4X_4 + b_5X_5 + b_6X_6 + e.$$

All tested variables were determined according to standard methods of soil chemical analysis (31).

Sulfide sorption by the clay fraction:

Kaolinite type 1:1 and Bentonite type 2:1 clays were used in this study. Samples weighing 0, 1, 2, 3, 4 or 5 g of a given clay were suspended in 100 ml of deionized distilled water. To each sample 1, 2, 3, 4, or 5 ml of Na_2S solution (equilivalent to 3, 6, 9, 12 or 15 ppm $[S^=]$, respectively) was added in the same order of clay fraction weight. Thus, a ratio of 3 ppm $[S^=]$ to 1 g clay was maintained. The sulfide electrode was used to detect the potential of the suspension while it was maintained at 24 $\pm$ 2 C under N_2 and stirred magnetically. The developed potential was recorded on the log scale or potential scale E (mV). The potential difference between the control (0 clay samples) and the other suspensions account for the sorption of $S^=$ ion. Then, the samples were centrifuged for 20 min at 27,000 g to remove the

physically adsorbed [$S^{=}$]. The activity of free $S^{=}$ in the supernatant accounted for the observed physical sorption. Results are expressed as ppm and percentages of $S^{=}$ sorbed or desorbed per g of clay.

Changes in H_2S concentration in culture solution with time:

Potentiometric titrations were used in this study to determine the changes in H_2S concentrations in culture solutions at 1, 3, 5, 6, 7, 8 and 18 hr in which no plants were included. Also, H_2S concentrations were determined for plant-containing culture solutions at 1, 3, 5, 6 and 7 hr with rice roots immersed in the culture solutions. Concentrations of H_2S used ranged from 0.173 to 8.810 ppm. Analyses of variance were run for treatments with and without plants and for combined treatments and the results are presented for some of the H_2S levels and time periods:

Pretreatment of plants with H_2S:

The Saturn cultivar of rice (Oryza sativa L.) was used in all experiments. Plants were grown in vermiculite, in 1-quart polyethylene containers at room temperature 24 $\pm$ 2 C for 12 hr in fluorescent light. The vermiculite was saturated with glass distilled water at time of planting and kept moist by adding one-fourth strength Hoagland's solution after sprouting. Seedlings 15-21 days old were selected for root uniformity. Rice plants were grouped into 30-40 seedlings per group, and roots of each group were fully immersed in solutions of known concentration of H_2S in long test tubes for 3, 5 or 6 hr as specified in each experiment. H_2S concentrations were considered high, medium or low at concentrations which ranged from 5-16, 4.8-1.6 and 3.0-0.1 ppm, respectively.

Following pretreatment with H_2S, the seedling roots were washed three times in distilled water and cut from plants under the water surface before use in studies of respiration.

Preparation of rice root homogenates:

Samples weighing 0.60 g from either non-treated or pretreated roots at lower levels of H_2S (3.2-.1 ppm) were homogenized for 2 min at top speed in a Sorvall Omni-mixer in 10 ml potassium phosphate buffer at an ionic strength and pH specified for each experiment. The homogenizing cup was immersed in an ice bath during grinding. The resulting homogenates were strained through four layers of cheesecloth and kept in an ice bath until used. For enzyme extracts, the homogenates were centrifuged at 1000 g at 4 C for 20 min and the supernatants were kept refrigerated prior to spectrophotometric studies.

The optimum concentration of substrate and tissue homogenate or enzyme extract used in each assay was determined by preliminary work. The substrates and tissue homogenates were prepared on the same day as the assays.

Determination of respiratory activity of the whole root:

Oxygen consumption was determined manometrically with a Gilson differential respirometer. All tests were carried out at 21 C. Duplicate flasks were run for all treatments, with air as the gas phase. Excised roots were floated in 3.0 ml 0.02 M potassium phosphate buffer at pH 6.0. The center well contained 0.3 ml of 10% KOH. Filter paper wicks were used in all center wells.

The first manometer reading was recorded after a 15 to 20 min equilibration period, and readings were taken at 15 min intervals. Oxygen consumption by root tissue is presented as μl/g dry wt/hr. Results are reported as average percent inhibition. The individual comparisons were made according to Duncan (20).

The same experiment was repeated using 0.01 M and 0.001 M KCN at 0.3 ml tipped from the side arm after 30 min.

Determination of enzymatic activity:

Enzymatic activities were estimated with a Perkin-Elmer recording spectrophotometer at a room temperature of 24 ± 2 C. The reference cuvette contained the same concentrations of the reaction mixture as the sample cuvette, except that the substrate solution was replaced with distilled water or a boiled enzyme extract. The enzymatic activities were estimated as the slopes of the linear portions of the curves obtained by plotting absorbance versus time and data recorded for representative experiments.

A. Iron-containing oxidases

Peroxidase activity was determined by measuring the oxidation of pyrogallol to purpurogallin in the presence of H_2O_2 at 425 mμ with slight modification of the methods of Gentile and Naylor (22), and Krupka (37). The sample cuvette contained 0.5 ml 0.1 M potassium phosphate buffer at pH 7.0, 0.3 ml enzyme extract, 0.3 ml 0.05 M pyrogallol, 0.1 M 1.0% H_2O_2, and distilled water to bring cuvette contents to 3.0 ml. A complete mixture containing boiled enzyme and another one lacking H_2O_2 served as controls.

Catalase activity was determined manometrically with a Gilson respirometer by measuring the evolution of oxygen in the presence of H_2O_2. A half-ml of homogenate was pipetted into the side arm. After equilibration, the contents of the side arm were tipped into the main compartment, which contained 3.0 ml of 0.01 M potassium phosphate buffer at pH 5.8 and 0.2 ml of 0.1% H_2O_2. Readings were taken at 1 min intervals for 5 min. Results were based on averages of two flasks as μl O_2/g fresh wt/hr.

Cytochrome oxidase activity was determined spectrophotometrically at 550 mμ by the change in optical density accompanying the oxidation of reduced cytochrome c by the enzyme extract (22). The reaction mixture contained 2.8 ml reduced cytochrome c solution (0.66 mg/ml) at pH 7.7 and 0.2 ml enzyme extract. The cytochrome c was reduced by adding sufficient sodium hydrosulfite (0.004 M) to obtain an absorbance ratio, A (550 mμ)/A (565 mμ), greater than 6.0 (40). Boiled enzyme extract served as control.

B. Copper-containing oxidases

Ascorbic acid oxidase activity was measured by following the disappearance of ascorbic acid at 265 mμ (40). The sample cuvette contained 1.0 ml potassium buffer at pH 7.0, 0.3 ml 0.01 M ascorbic acid, and 1.2 ml enzyme extract brought to a final volume of 3.0 ml with distilled water. The results were expressed as the changes in absorbance for the first 5 min of the reaction per 1.0 ml of extract.

Polyphenol oxidase activity was measured by following the oxidation of catechol at 546 mμ (38). The reaction mixture contained 1.0 ml 0.1 M potassium phosphate buffer at pH 7.0, 0.5 ml of

(10.0 mg/ml) catechol, 0.5 ml (5 mg/ml) proline (L), and 1.0 ml enzyme extract. The reaction mixture was incubated at room temperature for 15 min prior to reading. The enzyme activity was expressed as the change in absorption versus time (hr).

RESULTS

Response of the silver sulfide membrane electrode to sulfide ion activity and sample pH:

The calibration curve of the sulfide electrode was obtained with a series of solutions varying in $Na_2S.9H_2O$ concentrations but having a constant background of 1.0 M NaOH. The sodium hydroxide provided a fixed high pH, thus establishing a constant fraction of the total sulfide present in the form of free sulfide ion ($S^=$). In addition, the activity coefficient for $S^=$ was fixed by the high ionic strength ($\mu = 1.0$). The calibration curve (Fig. 1-A) gave a slope within $\pm$ .5 mV, with a mean of 30 mV of the 29.58 mV per activity decade of $S^=$ predicted by the Nernst equation at 25C (Eq. 7);

$$E = E^o - 29.58 \text{ Log. } {}^aS^= \quad \text{..... (Eq. 7)}$$

where

E = observed cell potential, mV

E^o = standard cell potential, mV

${}^aS^=$ = activity of sulfide ion, moles/liter

Millivolt readings E (mV) plotted against activity of ${}^aS^=$ on semilogarithmic graph paper yielded a straight line calibration curve with a theoretical slope of 29.58 mV/Log ${}^aS^=$. The slope of the calibration curve obtained in this study agrees with the Nernstian equation, even with $[S^=]$ as low as 10^{-6} M (.032064 ppm), (Fig. 1-A).

The experimental calibration curve was not easily extended below 10^{-6} M in sulfide ion concentrations by direct dilution due to

difficulty in preparing dilute sulfide solutions, even when precautions were taken to exclude air by purging them with oxygen-free nitrogen. However, the activity of free sulfide ion in solution may be controlled with the presence of complexing agents such as the hydrogen ion, as shown in Eqs. 8 and 9.

$$S^{=} + H^{+} \rightleftharpoons HS^{-} \quad \text{..... (Eq. 8a)}$$

$$K_2 = \frac{a_{S^{=}} \cdot a_{H^{+}}}{a_{HS^{-}}} \quad \text{..... (Eq. 8b)}$$

$$HS^{-} + H^{+} \rightleftharpoons H_2S \quad \text{..... (Eq. 9a)}$$

$$K_1 = \frac{a_{H^{+}} \cdot a_{HS^{-}}}{a_{H_2S}} \quad \text{..... (Eq. 9b)}$$

The equilibrium thus established made possible an extension of the calibration curve down to a concentration of 10^{-20} M in $S^{=}$ ion. Such an extended calibration curve is shown in Fig. 1-B. Although the total sulfide present in the system is essentially constant, the potential of the silver sulfide membrane electrode is a function of the free sulfide ion activity ($^{a}S^{=}$) (Eq. 7). This activity is in turn governed by the pH - dependence of the equilibria in Eqs. 8a and 9a.

The dependence of the electrode potentials on the pH and the sulfide ion activity is shown in Fig. 1-C. The concentration of $Na_2S.9H_2O$ used was $.92 \times 10^{-3}$ M $S^{=}$. The pH value was varied from 12.5 to 5.0 by the use of HCl or borate buffer solutions (28), while the ionic strength ($\mu = 1.0$) was kept constant. The potential of the sulfide electrode versus hydrogen ion activity ($^{a}H^{+}$) showed a uniform experimental slope of 30 ± 0.5 mV per pH unit, in agreement with that predicted by the Nerstian equation (Eq. 10).

$$E = E \quad - 29.6 \text{ pH} \quad \text{..... (Eq. 10)}$$

Figures 1-A and 1-C show that the sulfide electrode responds to the H^+ activity in a reversible manner as well as to the $S^=$ activity in sulfide electrolytes.

The silver-sulfide membrane electrode was also used to determine a potentiometric titration mode as an end-point detector for sulfide ion. Fig. 1-D typifies a titration curve of standard sulfide solution obtained by using the sulfide electrode to follow the addition of $AgNO_3$ standard solutions.

Sulfide-electrode response to sulfide ion concentrations:

Concentration measurements were made using standardizing solutions with the same ionic strength ($\mu = .10$). The method for measuring concentrations was the same as for measuring activity. Fig. 2-B shows the calibration curve of sulfide ion concentration $S^=$ versus the sulfide electrode potential E (mV) as measured on the logarithmic scale. The electrode potential responded in the same manner as the sulfide concentration, as shown in Fig. 2-B. Figure 2-C shows a calibration curve of the sulfide ion concentration versus the sulfide electrode potential. The electrode exhibited a 30 ± 0.5 mV change in potential for each ten-fold change in sulfide ion concentration. It should be noted that in the very dilute solutions the activity coefficient equals unity and consequently the concentration of the sulfide ion will be equal to the activity as shown by equation 11;

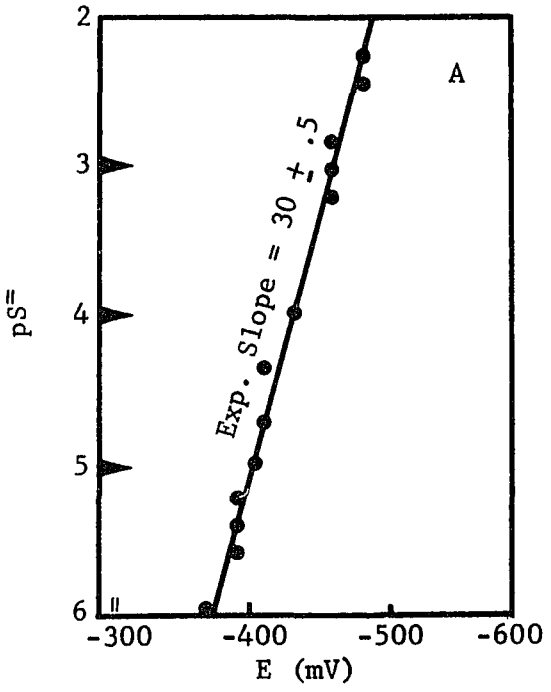
$$C_t = f(^aS^=) \quad \text{.....(Eq. 11)}$$

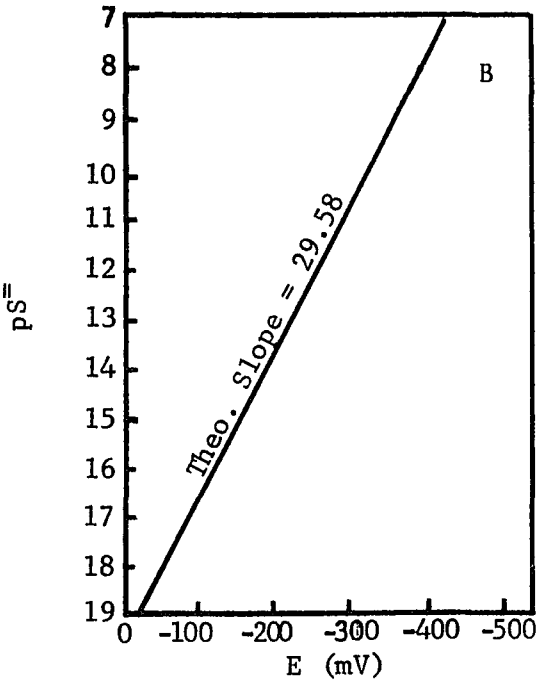
where C_t = total sulfide ion concentration

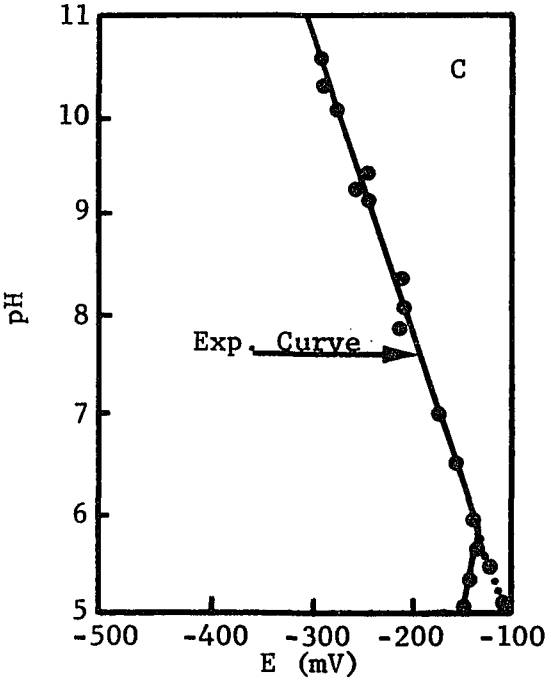
f = activity coefficient

$^aS^=$ = sulfide ion activity

Figure 1. Response of silver sulfide membrane electrode to sulfide and hydrogen ion activities. A) Calibration curve for sulfide ion ($S^{=}$ in 1.0 M NaOH, B) Extended calibration curve for sulfide ion in 1.0 M NaOH, C) Calibration curve as a function of pH at 32 ppm $S^{=}$, D) Potentiometric titration of 100 ml of .00092 M-Na_2S ($S^{=}$ = 29.5 ppm) with 0.1 M $AgNO_3$. Potential measured vs. reference electrode Ag/AgCl (0.1 M) with KNO_3 salt bridge.


A
Exp. Slope = 30 ± .5
pS=
E (mV)
2
3
4
5
6
-300
-400
-500
-600


B
Theo. Slope = 29.58
pS=
E (mV)
7
8
9
10
11
12
13
14
15
16
17
18
19
0
-100
-200
-300
-400
-500


C
Exp. Curve
pH
E (mV)
11
10
9
8
7
6
5
-500
-400
-300
-200
-100

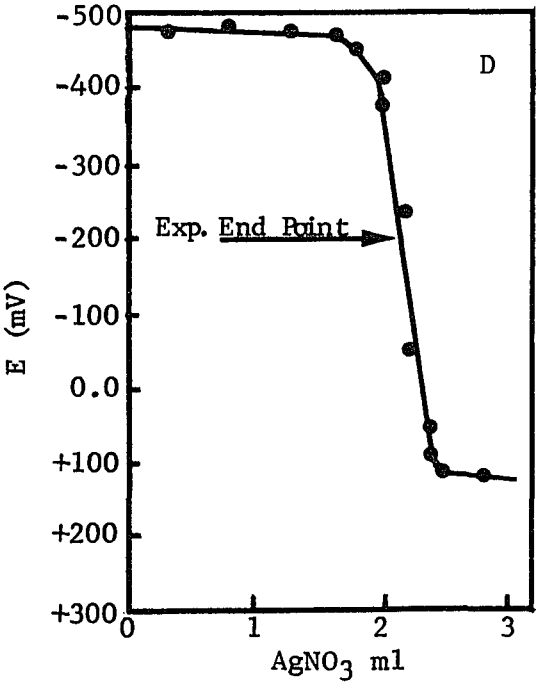
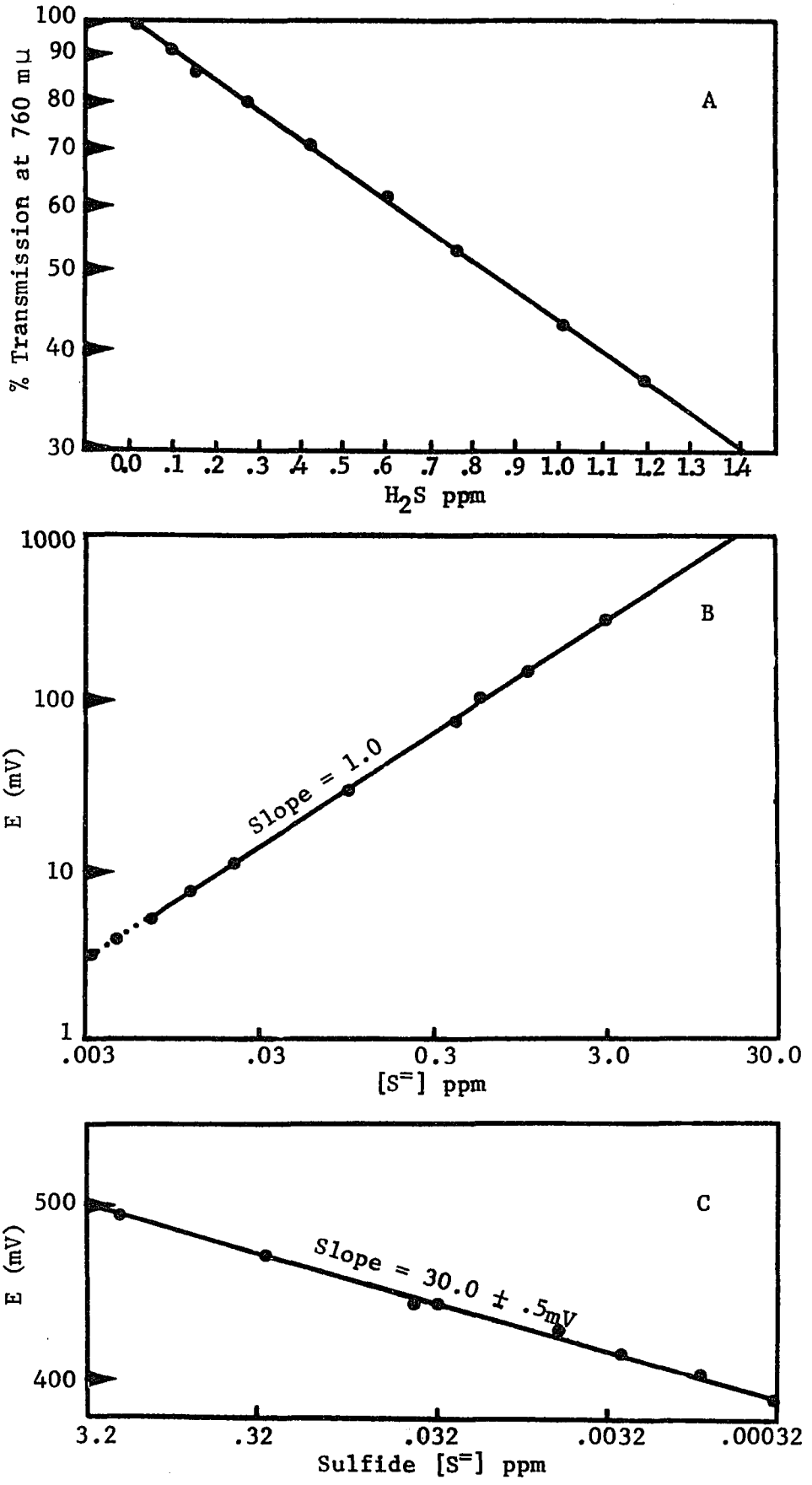

D
Exp. End Point
E (mV)
AgNO3 ml
-500
-400
-300
-200
-100
0.0
+100
+200
+300
0
1
2
3

Figure 2. H_2S concentration as measured by the methylene blue method (67) and the sulfide electrode. A) Calibration curve for sulfide-sulfur determined by the methylene blue method, B) Logarithimic plot of sulfide electrode potential E (mV) vs. ppm $S^=$ concentration, C) Semi-logarithimic plot of $S^=$ concentration in ppm vs. the sulfide electrode potential E (mV).


A
% Transmission at 760 mμ
100
90
80
70
60
50
40
30
0.0 .1 .2 .3 .4 .5 .6 .7 .8 .9 1.0 1.1 1.2 1.3 1.4
H2S ppm
B
E (mV)
1000
100
10
1
Slope = 1.0
.003
.03
0.3
3.0
30.0
[S=] ppm
C
E (mV)
500
400
Slope = 30.0 ± .5mV
3.2
.32
.032
.0032
.00032
Sulfide [S=] ppm

The methylene blue method gave results very close to those obtained with the sulfide electrode. A calibration curve of H_2S in ppm determined by the methylene blue method is shown (Fig. 2-A). The correlation coefficient ($r = .9965$) between the two methods was highly significant at the .001 level of probability (Fig. 3).

Survey of H_2S levels in Louisiana rice fields:

The amounts of hydrogen sulfide calculated on the basis of free $S^=$ ions in 53 different sites in Louisiana rice fields are shown in Table 2. Also, the site pH and Eh_7 values were reported on a bi-weekly basis during the end of tillering and the beginning of the booting stage (July 10), during the heading-flowering stage (July 24), and during the ripening stage (August 5).

Measurable amounts of free H_2S accumulated in the soils. The mean for the 53 sites was .10373 ppm H_2S. Sixteen sites showed H_2S in amounts greater than 0.1 ppm, while the other sites showed amounts less than .07 ppm H_2S. Amounts of H_2S ranged from .00025 to .10138, from .00004 to .64126, and from .000006 to .40569 ppm, with averages of .02099, .175604, and .104639 ppm H_2S for the booting, heading-flowering, and ripening stages, respectively (Table 2).

The pH values showed a significant decrease (Table 3) with time ranging from pH 6.9 - 6.3 at booting, 6.5 - 5.4 at heading-flowering, to 6.3 - 5.4 at the ripening stage. The mean pH levels were 6.67, 6.15 and 5.92, respectively. Redox-potentials (Eh_7) ranged from -50 to +36 mV, with a mean of 4.7 mV at booting; from -208 to +92 mV with a mean of -50.25 at heading-flowering; and from -87 to +13 with a mean of -37 at the ripening stage. The changes in pH, Eh and $pS^=$ values were significant from one field to another within weeks as shown in Table 3.

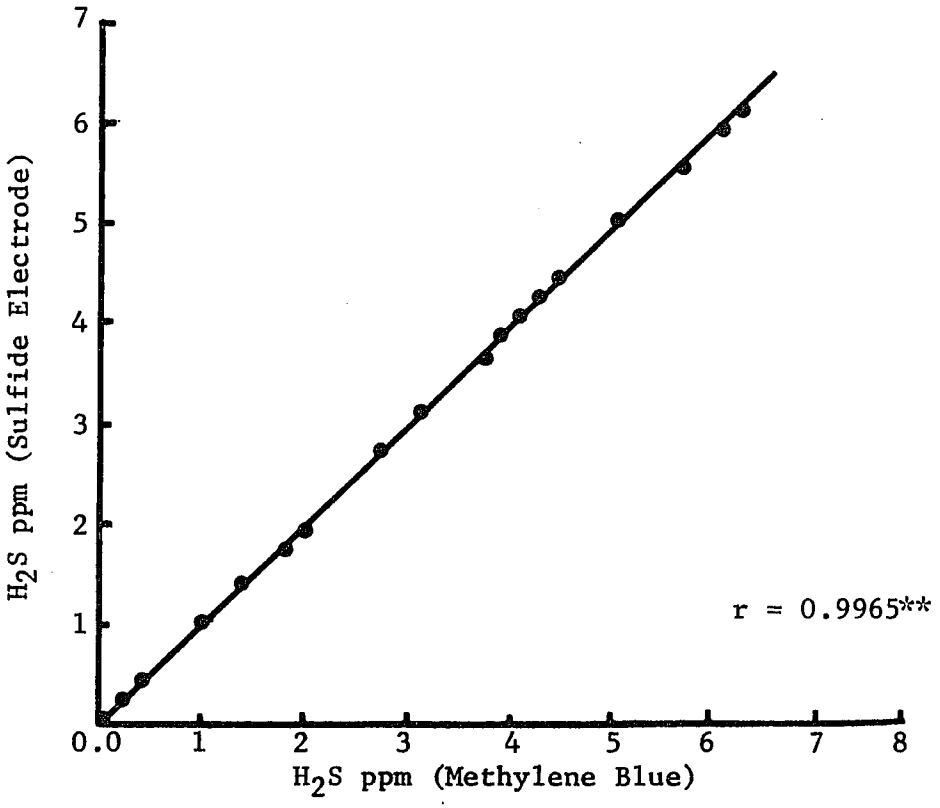


Figure 3. Bivariant graph of H_2S concentrations in ppm determined by the methylene blue method and the sulfide electrode.

Table 2. Free hydrogen sulfide concentrations determined during July 10 - August 5, 1970 with a sulfide electrode at field pH and Eh_7 for 25 Louisiana rice fields.

Sampling date	Field #	Site	pH	Eh mV[7]	H_2S ppm
July 10, 1970	1	a	6.5	-6	.00025
End of tillering	1	b	6.6	-50	.00161
and beginning of booting stage	2	a	6.3	-8	.00159
	2	b	6.4	2	.03399
	2	c	6.5	25	.03400
	3	a	6.8	36	.03517
	3	b	6.7	16	.01013
	3	c	6.8	-5	.01084
	4	a	6.9	22	.01013
	4	b	6.9	16	.05216
	4	c	6.9	25	.01817
	5	a	6.7	-14	.10138
	5	b	6.8	2	.05216
	Average		6.67	+4.7	.02099
July 24, 1970	6	a	6.2	10	.00101
Heading-flowering stage	6	b	6.2	10	.00101
	7	a	6.8	92	.00064
	7	b	6.4	-12	.00004
	8	a	5.8	-123	.64128
	8	b	5.8	-168	.64128
	9	a	6.5	2	.64128
	9	b	6.5	-208	.50980
	10	a	6.4	-167	.00405
	10	b	6.3	4	.00641
	10	c	6.2	24	.01643
	11	a	6.3	-8	.00641
	11	b	6.2	16	.01643
	12	a	5.6	-72	.00456
	13	a	5.6	-106	.25490
	13	b	5.8	-98	.06412
	Average		6.15	-50.25	.17560

Table 2 (continued). Free Hydrogen sulfide concentrations determined during July 10 - August 5, 1970 with a sulfide electrode at field pH and Eh_7[1] for 25 Louisiana rice fields.

Sampling date	Field #	Site	pH	Eh mV[7]	H_2S ppm
August 5, 1970	14	a	6.2	+3	.00051
Ripening stage	14	b	5.9	-45	.01603
	15	a	6.3	-11	.00051
	15	b	6.3	-1	.00051
	16	a	6.0	-59	.12825
	16	b	6.1	-33	.06428
	17	a	6.2	-57	.00005
	17	b	6.2	+3	.00016
	18	a	6.0	+11	.00001
	18	b	6.2	-87	.01016
	19	a	5.9	-55	.40561
	19	b	6.0	-59	.05098
	20	a	5.9	-35	.04056
	20	b	5.9	-25	.04056
	21	a	5.9	-15	.00405
	21	b	5.9	-5	.04056
	22	a	6.2	+13	.10164
	22	b	6.0	-49	.05981
	23	a	5.6	-53	.16032
	23	b	5.6	-43	.16032
	24	a	5.6	-63	.16032
	24	b	5.6	-83	.25490
	25	a	5.4	-55	.40589
	25	b	5.4	-85	.40569
	Average		5.92	-37	.10464

[1] Eh_7 = 241 + Eh observed + (7-pH observed)(-60mV)

Table 3. Analysis of variance for pH, Eh[1] and $pS^{=}$[2] for sulfide sampling sites nested within fields, within weeks.

Source of Variation	d.f.	M. S.		
		pH	Eh	$pS^{=}$
Weeks	2	3.039**	2272.527	2731.470
Fields/W	22	0.210**	2904.031*	2346.014**
Sites/F/W (Error)	28	0.014	1214.655	536.206

1 No conversion to Eh_7 was used in the statistical analysis, hence its effect on the variance is constant.

2 No transformation was made from $pS^{=}$ to H_2S.

* Significant at the .05 level of probability.

** Significant at the .01 level of probability.

Measured amounts of H_2S during heading-flowering stage (July 24) were compared with theoretical values of H_2S (Table 4). The measured H_2S values differed significantly ($P < .005$) from the theoretical H_2S values, being 3 to 10,000-fold higher than the maximum limits of theoretically predicted values in all cases, except in one field (#2). In five fields (1, 4, 5, 6 & 8) measured H_2S values ranged from 3 to 50 times higher than the theoretical maximum, with an average of 30-fold higher. In one field H_2S values were 10,000 times higher than the theoretical maximum. In another field (#7), the H_2S values were 300 times higher, and in a third field (#2) they were within the range of theoretical prediction. At site (a) in field #2 the measured H_2S values were intermediate between the theoretical minimum and maximum limits predicted (Table 4).

Regression of H_2S on Fe^{++}, Mn^{++}, oxidizable carbon, clay percentage, Eh_7 and pH.

The regression of H_2S concentrations on the six independent variables was significant. However, certain independent variables contributed very little to explaining the variation in H_2S (Table 5).

Soil pH (X_4) alone accounted for 71.5% of the variation in H_2S. Including manganese (X_6) gave an increase in explained variation of 3.5% while adding oxidizable carbon (X_2) increased the explained variation by an addition of 3.5% and bringing in clay percent (X_2) gave a further improvement of 1.4%, all of which caused significant improvement. However, ferrous concentration (X_1) and redox-potential (X_5) did not significantly improve the explanation of variation in H_2S concentration (Table 5).

Table 4. Measured[1] and theoretical values of H_2S in ppm.

Field #	Sites	pH	Measured H_2S ppm.	Theoretical H_2S ppm.[2] Minimum	Maximum
1	a	6.2	$.101 \times 10^{-2}$	$.82 \times 10^{-5}$	$.328 \times 10^{-3}$
	b	6.2	$.101 \times 10^{-2}$	$.82 \times 10^{-5}$	$.328 \times 10^{-3}$
2	a	6.4	$.405 \times 10^{-4}$	$.33 \times 10^{-5}$	$.131 \times 10^{-3}$
	b	6.8	$.640 \times 10^{-3}$	$.50 \times 10^{-6}$	$.210 \times 10^{-4}$
3	a	6.5	.641	$.21 \times 10^{-5}$	$.820 \times 10^{-4}$
	b	6.5	.509	$.21 \times 10^{-5}$	$.509 \times 10^{-4}$
4	a	6.4	$.405 \times 10^{-2}$	$.33 \times 10^{-5}$	$.131 \times 10^{-3}$
	b	6.3	$.641 \times 10^{-2}$	$.52 \times 10^{-5}$	$.207 \times 10^{-3}$
	c	6.2	$.164 \times 10^{-1}$	$.82 \times 10^{-5}$	$.328 \times 10^{-3}$
5	a	5.6	.254	$.33 \times 10^{-3}$	$.131 \times 10^{-1}$
	b	5.8	$.641 \times 10^{-1}$	$.52 \times 10^{-4}$	$.207 \times 10^{-2}$
6	a	5.4	$.456 \times 10^{-2}$	$.33 \times 10^{-4}$	$.131 \times 10^{-2}$
7	a	5.8	.641	$.52 \times 10^{-4}$	$.207 \times 10^{-2}$
	b	5.8	.641	$.52 \times 10^{-4}$	$.207 \times 10^{-2}$
8	a	6.3	$.641 \times 10^{-2}$	$.52 \times 10^{-5}$	$.207 \times 10^{-3}$
	b	6.2	$.164 \times 10^{-1}$	$.82 \times 10^{-5}$	$.382 \times 10^{-3}$
χ^2				3,145.272**	481.551**

[1] Measured in the field (July 24, 1970) during heading-flowering stage.

[2] Maximum limits $pH_2S = 2pH - 4.40$
Minimum limits $pH_2S = 2pH - 2.81$

** Significant at the 0.005 level of probability.

Table 5. Regression equations and coefficients of determination (R^2) for regression of H_2S concentrations on various independent variables.

Independent variables[1]	H_2S concentrations as a function of independent variables	R^2
$X_1X_2X_3X_4X_5X_6$		.824
$X_2X_3X_4X_5X_6$		.818
$X_2X_3X_4X_6$	$H_2S = 2.69 - .02X_2 + .002X_3 - 0.43X_4 - 0001X_6$	.809*
$X_2X_4X_6$	$H_2S = 2.51 - .024X_2 - 0.39X_4 - .0001X_6$	.785**
X_4X_6	$H_2S = 2.57 - 0.411X_4 - .0001X_6$	.750**
X_4	$H_2S = 2.45 - 0.39X_4$	.715**

1 $X_1 = Fe^{++}$ ppm, X_2 = oxidizable carbon %, X_3 = clay %, X_4 = pH, $X_5 = Eh_7$, $X_6 = Mn^{++}$ ppm.

* Denotes significant increase in R^2 over lower R^2 at the .05 level of probability.

** Denotes significant increase in R^2 over lower R^2 at the .01 level of probability.

Sorption and desorption of $S^{=}$ by the clay fraction:

Table 6 shows the results of sorption and desorption of $S^{=}$ by type 2:1 bentonite clay. This clay showed $S^{=}$ sorption in the range of 2.82-2.68 ppm H_2S per g of clay dry weight. The sorped $S^{=}$ ratio to clay weight was 2.70 ppm $S^{=}$/1 g bentonite, while the total sorped $S^{=}$ amount was proportional to the amount of clay (Table 6). Recovery of desorped $S^{=}$ by centrifugation accounted for .021 to .011 ppm $S^{=}$. Also, there were about 2.9% and 5% loss of $S^{=}$ into the atmosphere from the pure Na_2S solution and the Na_2S suspension of clay, respectively. No sorption by kaolinite type 1:1 clay was apparent when the experiment was repeated.

The pH reaction of clay suspensions ranged 9.5-9.8 and 10.3-10.7 for bentonite and 7.6-8.4 and 10.2-10.5 for kaolinite before and after addition of Na_2S solutions. Therefore, the sorption of $S^{=}$ by the two types of clay was due to their physiochemical characteristics and not to the sample pH. The added amounts of $S^{=}$ were on the order of 6×10^{-5} M and were so small that conversion from activities to concentrations was unnecessary.

Changes in H_2S concentration in culture solution with time:

The H_2S concentrations in culture solutions (in which plants or no plants were included) were measured and found to decrease with time, as shown for some H_2S levels and four periods of exposure (Table 7). There was not a significant difference in the rate of H_2S loss between cultures containing plants and cultures in which no plants were included. However, a highly significant difference was apparent for H_2S concentration, period of exposure, and all first and second order interactions, except for the plant x concentration interaction (Table 8).

Table 6. Sorption and desorption of $S^{=}$ by bentonite clay at pH 10.5 - 11.8.

Sample gm.	$S^{=}$ Added ppm	Sample Basis		One Gram Basis	
		$S^{=}$ Sorped ppm	$S^{=}$ Recovered ppm	$S^{=}$ Sorped %[1]	$S^{=}$ Recovered %[2]
		Clay Samples			
1	3	2.71	.015	90.33	5.00
1	3	2.76	.014	92.00	4.66
1	3	2.74	.011	91.33	3.66
1	3	2.74	.011	91.33	3.66
2	6	5.62	.029	93.66	4.66
2	6	5.61	.030	93.33	5.00
2	6	5.64	.032	94.00	5.33
2	6	5.64	.031	94.00	5.00
3	9	8.31	.051	92.33	5.66
3	9	8.31	.052	92.00	5.66
3	9	8.32	.050	92.33	5.33
3	9	8.33	.047	92.33	5.00
4	12	10.84	.081	90.33	6.66
4	12	10.74	.080	89.33	6.66
4	12	10.81	.084	90.00	7.00
4	12	10.79	.084	90.00	7.00
5	15	13.55	.104	90.33	6.66
5	15	13.47	.100	89.66	6.66
5	15	13.47	.100	90.33	6.66
5	15	13.56	.102	90.33	6.66
		Control Samples			
0[2]	3	2.92	2.90	97.33	96.66
0	3	2.90	2.90	96.66	96.66
0	3	2.94	2.91	98.00	97.00
0	3	2.91	2.89	97.00	96.33

[1] Sulfide concentration = $S^{=}$ added - measured $S^{=}$ in ppm.

[2] Percent of initial $S^{=}$ added.

Table 7. H_2S[1] changes in culture solution with or without rice seedlings (plants) as a function of some exposure periods and concentrations.

H_2S Concentration (ppm) and %[2]	Without Plants				With Plants			
	Time (hr)				Time (hr)			
	1	3	5	6	1	3	5	6
8.81	5.73	5.71	5.61	5.52	8.01	6.18	5.83	5.59
100%	65.0	64.8	63.6	62.3	90.9	70.1	66.1	63.4
3.52	1.49	1.28	1.12	1.10	3.32	1.78	1.35	1.19
100%	42.5	36.3	31.8	31.2	94.3	50.5	38.3	33.8
0.71	0.51	0.32	0.29	0.28	0.54	0.33	0.31	0.28
100%	71.8	45.0	40.8	40.4	76.0	46.4	43.6	39.4
0.17	0.12	0.11	0.10	0.09	0.13	0.12	0.11	0.09
100%	70.5	64.7	58.8	52.9	76.4	70.5	64.7	52.9
Overall Mean								
2.90	1.77	1.64	1.51	1.42	2.70	1.86	1.54	1.45
100%	61.0	56.5	52.0	48.9	84.4	64.1	53.1	50.0

[1] Average of two readings.

[2] Percent (%) of concentration at initial application.

Table 8. Analysis of variance of average change in H_2S concentrations in culture solution, with and without plants, as a function of exposure time.

Source of Variation[1]	d.f.	M.S.
Treat (Plant)	1	2.849
Time	5	12.592**
Conc.	9	116.234**
Plant x Time	5	1.268**
Plant x Conc.	9	0.401
Time x Conc.	45	1.196**
Plant x Time X Conc.	45	0.209**
Error	120	0.00000019
Total	239	

[1] Plants were considered as fixed effects.

** Significant at the .01 level of probability.

Effect of H_2S concentration and contact period on rice root respiration:

The percent inhibition of respiration in H_2S-pretreated rice roots increased in a cumulative manner in relation to the duration of the contact period (Table 9). However, this inhibition was linear over the first four hr of exposure, after which it decreased with time (Table 9).

Change in respiration percent inhibition was significant at all exposures except at the first 1 hr period. Critical inhibition of respiration was apparent at 1, 3, 4 and 5 hr after contact with H_2S.

Respiration percent inhibition of H_2S-pretreated rice roots with high, medium and low levels of H_2S for 5 hr in water culture is shown in Table 10. The percent inhibition of oxygen consumption was about 40%, 25% and 14% at initial concentrations of 16.0, 3.2 and 0.1 ppm H_2S, respectively. Respiration percent inhibition by low levels of H_2S was significant at almost all concentrations when compared with its control. No respiration percent inhibition was significant between the two highest concentrations at each level, i.e., 16.0-10.0, 4.8-3.0 and 3.2-0.5 ppm for high, medium and low levels of H_2S, respectively.

Inhibition was cumulative and was increased by increasing H_2S concentration, except at 4.8 ppm H_2S. This overall linear increase was significantly proportional to H_2S concentrations at the high and low levels as shown by Duncan's multiple range test (Table 10).

Rice root respiration was 50 to 60% inhibited when treated with potassium cyanide at a final concentration of 0.001 M (26.017 ppm CN) (Fig. 4) and this inhibition was statistically significant.

Table 9. Effect of contact period on respiration of H_2S-pretreated rice roots at 3 ppm H_2S.

Contact Period (hr)	Mean % inhibition O_2 µl/hr/g dry weight (at 3.0 ppm H_2S)	Conclusion*
0.0	0.0	a
1.0	9.6	a
2.0	12.3	ab
3.0	20.8	bc
4.0	29.5	c
5.0	26.8	bc

* All means which are followed by a letter in common do not differ significantly from each other at the .05 level of probability.

Table 10. Effect of H_2S concentration on respiration of H_2S-pretreated rice roots for 5 hr.

H_2S Concentration (ppm)	Mean Oxygen Uptake µl/hr/g dry weight	Inhibition % of control	Conclusion*
16.0	2423.39	39.7	a
10.0	2697.45	32.5	ab
5.0	3343.68	16.3	b
0.0	3391.14	00.0	b
4.8	2888.35	22.3	a
3.0	2715.47	26.0	a c
1.6	3342.27	10.4	b
0.0	3734.00	00.0	b
3.2	2828.80	25.6	a
0.5	3183.33	16.7	ab
0.1	3288.09	14.0	b
0.0	3820.24	00.0	c

* All means which are followed by a letter in common do not differ significantly from each other at the .05 level of probability.

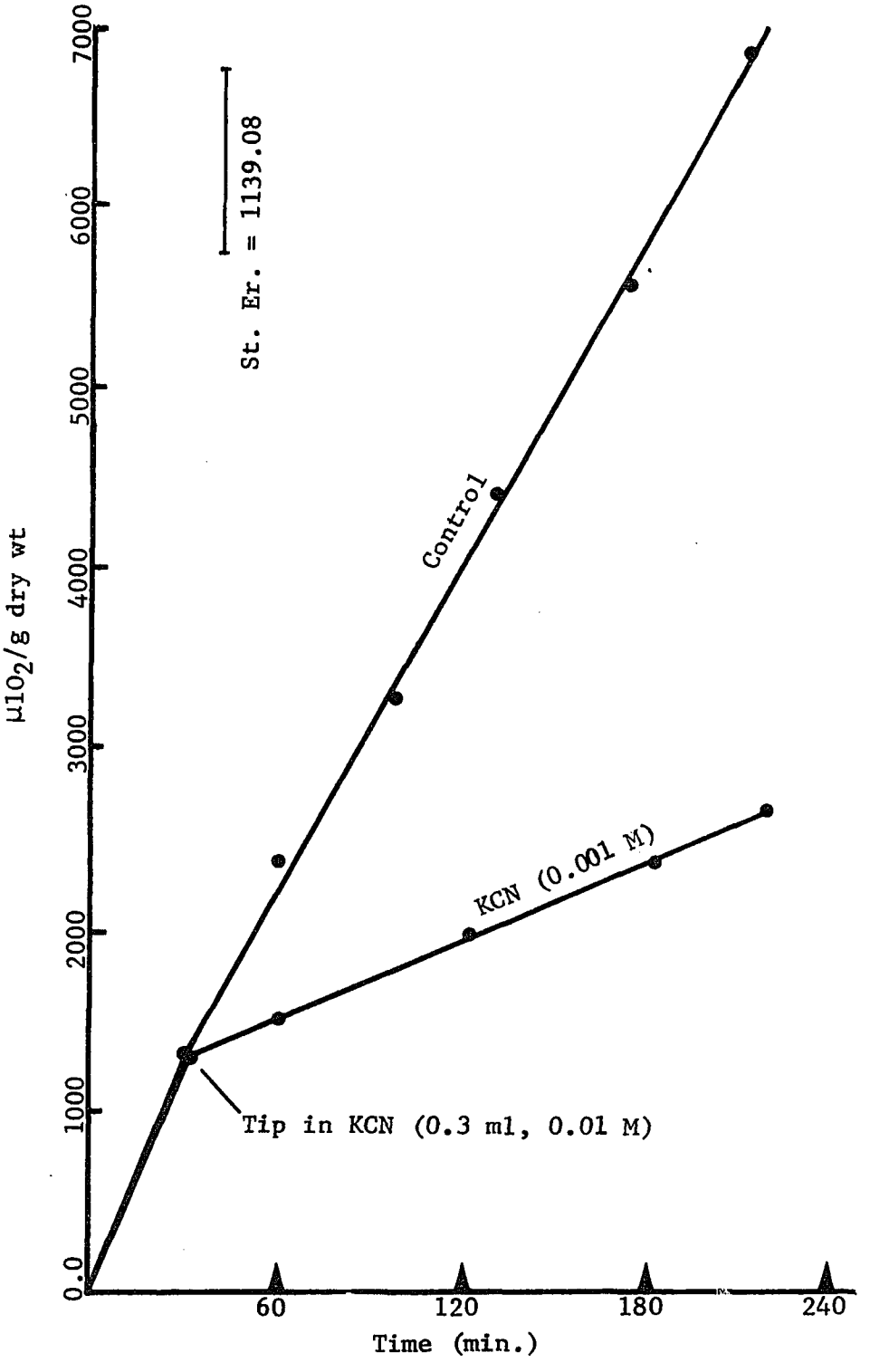


Figure 4. Response of rice root respiration to the addition of 0.001 M KCN.

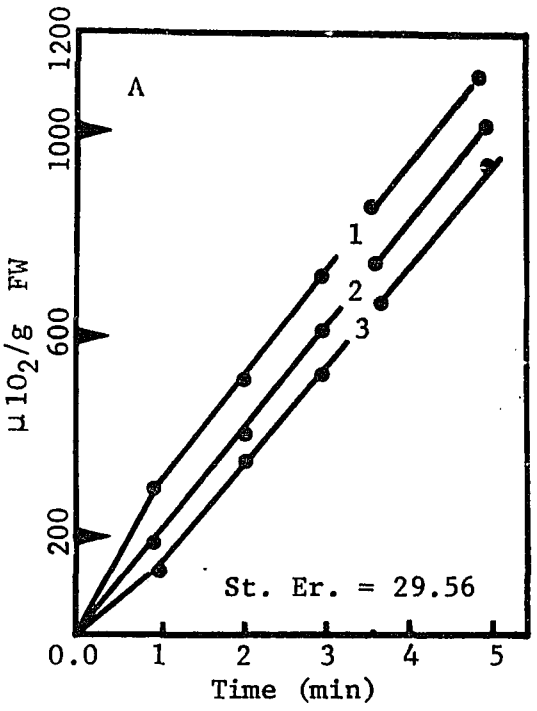
Effects of H_2S on enzymatic activities:

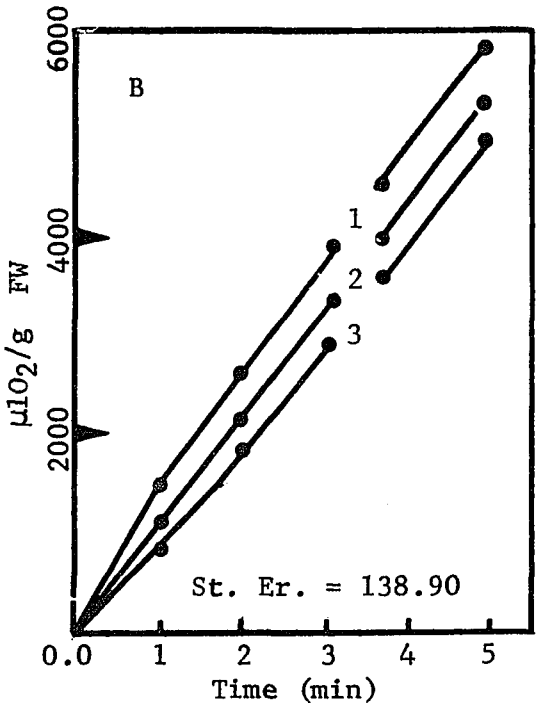
A survey of the literature revealed that alteration of the enzymatic activity of plant tissue occurs as a result of pathological, enviromental, or physiological stress conditions. These reports, although primarily concerned with terminal oxidases, have also included alterations in catalase and peroxidase activities. Due to our findings on sulfide levels and the effect of respiratory inhibitors found by the Japanese workers on rice root respiration and oxidative capacity, it was decided to investigate the effect of H_2S on root oxidases, especially catalase and peroxidase activities in H_2S-pretreated rice seedlings.

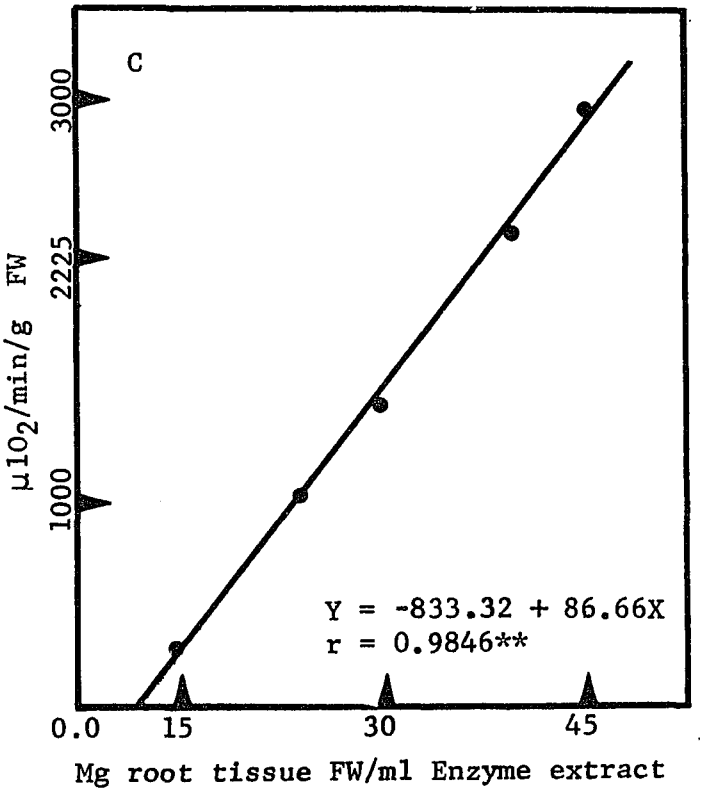
A. Catalase:

Catalase activity in roots at a root concentration of 15 mg tissue/ml homogenate, was inhibited 28 $\pm$ 2.35% and 22 $\pm$ 2.35% at 2.4 and 1.2 ppm of H_2S, respectively, after an initial 5 hr pretreatment (Fig. 5-A). In further experiments, the percent inhibition of catalase activity was relatively the same, despite the use of a more concentrated homogenate. Fig. 5-B shows that catalase activity, measured in 30 mg root tissue/ml tissue homogenate, was 27 $\pm$ 2.23 and 20 $\pm$ 2.23% inhibited at H_2S concentrations of 2.4 and 1.2 ppm, respectively, after an initial 5 hr pretreatment. Statistical analysis of these results showed significant inhibition in all cases. Catalase concentration revealed by activity measurements (Fig. 5-C) was correlated significantly ($r = 0.9846$) with mg/ml of fresh root tissue in a given tissue homogenate as was expected.

Figure 5. Effect of H_2S concentrations (1 = 0.0, 2 = 1.2 and 3 = 2.4 ppm) on catalase activity of pretreated rice roots for a 5 hr period. A) H_2O_2 = 0.1% and 15 mg root tissue/ml tissue homogenate, B) H_2O_2 = 0.1% and 30 mg root tissue/ml tissue homogenate, C) Catalase activity in relation to root extract concentration.


A
1200
1000
600
200
$\mu lO_2/g$ FW
1
2
3
St. Er. = 29.56
0.0
1
2
3
4
5
Time (min)


B
6000
4000
2000
$\mu lO_2/g$ FW
1
2
3
St. Er. = 138.90
0.0
1
2
3
4
5
Time (min)


C
3000
2225
1000
$\mu lO_2/min/g$ FW
Y = -833.32 + 86.66X
r = 0.9846**
0.0
15
30
45
Mg root tissue FW/ml Enzyme extract

B. Peroxidase:

Substantial inhibition of peroxidase activity occurred as a function of H_2S concentration and contact period (Fig. 6-A, 6-B). Percent inhibition of peroxidase activity at a 3 hr pretreatment was, on the average, 64% of that at a 6 hr pretreatment (Table 11). Percent inhibition was 7.7 and 32.2 after an initial 3 hr pretreatment with H_2S at 0.1 and 3.2 ppm, respectively, while it was 10.7 and 55.4 after an initial 6 hr pretreatment with H_2S at 0.1 and 3.2 ppm, respectively (Table 11).

C. Copper-containing enzymes:

Ascorbic acid oxidase activity was inhibited 29.0% and 40.0% at concentrations of 0.1 and 3.2 ppm H_2S, respectively after an initial 6 hr pretreatment (Fig. 6-C, Table 11). Percent inhibition of polyphenol oxidase activity was 7.8 and 37.7% at H_2S concentrations of 0.1 and 3.2 ppm, respectively after a 6 hr pretreatment (Fig. 6-D, Table 11).

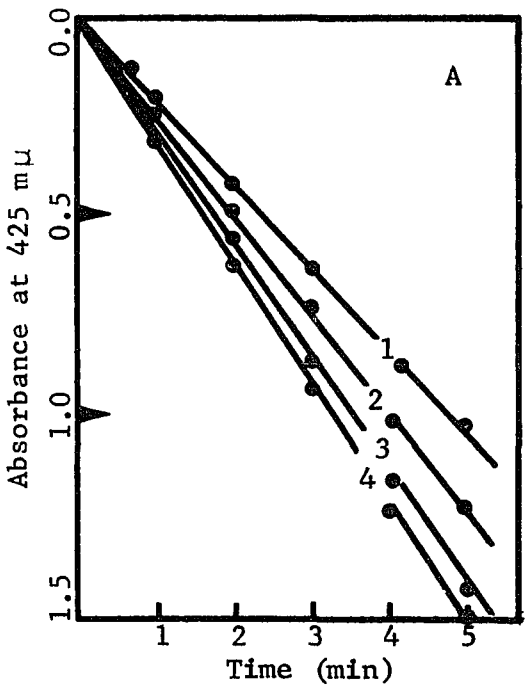
Figure 6. Effect of H_2S concentrations (1 = 3.2, 2 = 0.7, 3 = 0.1 and 4 = 0.0 ppm) on Peroxidase, Ascorbic Acid Oxidase and Polyphenol Oxidase.

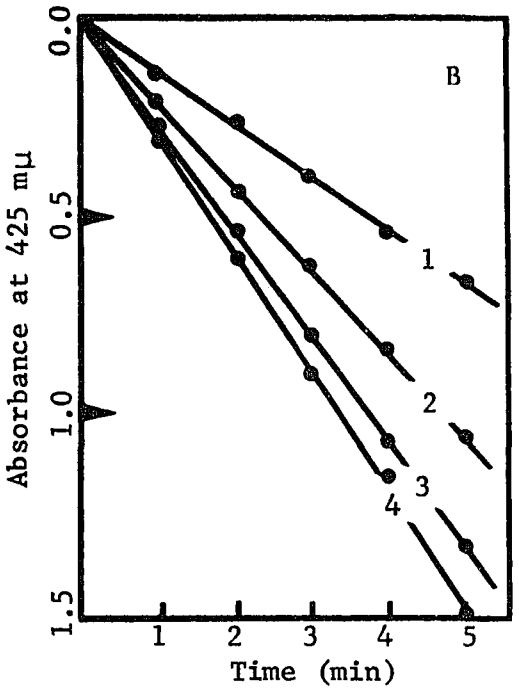
A) Peroxidase - 3 hr pretreatment.

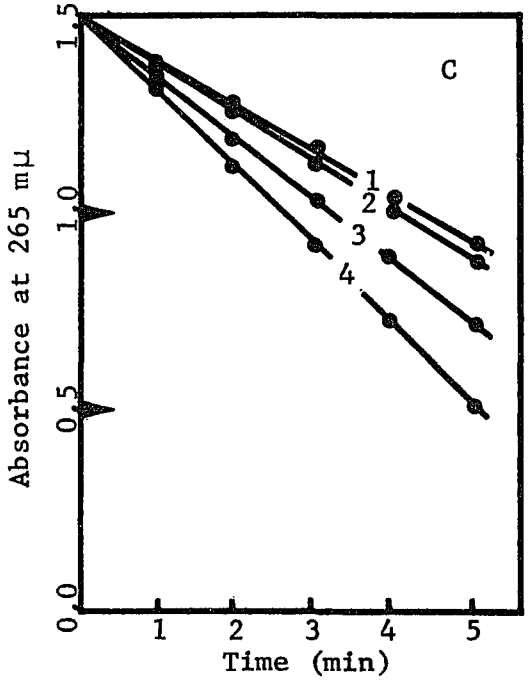
B) Peroxidase - 6 hr pretreatment.

C) Ascorbic Acid Oxidase - 6 hr pretreatment.

D) Polyphenol Oxidase - 6 hr pretreatment.


A
Absorbance at 425 mμ
0.0
0.5
1.0
1.5
1
2
3
4
5
Time (min)
1
2
3
4


B
Absorbance at 425 mμ
0.0
0.5
1.0
1.5
1
2
3
4
5
Time (min)
1
2
3
4


C
Absorbance at 265 mμ
0.0
0.5
1.0
1.5
1
2
3
4
5
Time (min)
1
2
3
4

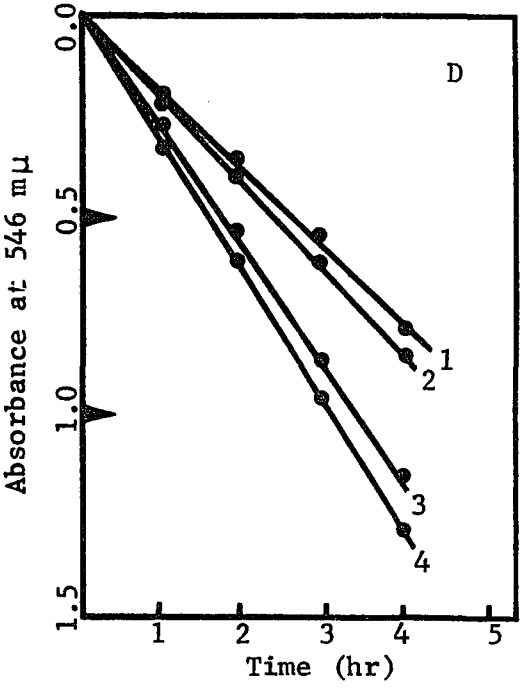

D
Absorbance at 546 mμ
0.0
0.5
1.0
1.5
1
2
3
4
5
Time (hr)
1
2
3
4

Table 11. Effect of H_2S concentrations on peroxidase, ascorbic acid oxidase and polyphenol oxidase activities of H_2S pretreated rice roots.

Percent inhibition of activity after hours of pretreatment with different concentrations of H_2S.

H_2S Concentration (ppm)	Peroxidase		Ascorbic Acid Oxidase	Polyphenol Oxidase
	3 hr	6 hr	6 hr	6 hr
0.0	0.0	0.0	0.0	0.0
0.1	7.7	10.7	29.0	7.8
0.7	18.5	29.8	38.0	33.3
3.2	32.2	55.4	40.0	37.7

DISCUSSION

The experimental results indicated that the sulfide electrode can be used to determine either the activity of free sulfide ion or the total concentration of soluble sulfides. The electrode method was preferred to the methylene blue method because it is more practical and convenient for in situ measurements as well as for low limits of $S^{=}$ detection. According to Ross (66) and others (16, 19, 28, 71), free sulfide ion levels on the order of 10^{-19}M are easily determined in acid solutions. Although these conclusions are supported by the limited data obtained in the present work, it will be necessary for workers to obtain large numbers of $S^{=}$ determinations in various media including sewage (19), factory effluents (71), and the soil and water of marshes and sea coasts, in order to confirm the occurrence and assess the importance of extremely low soluble sulfide concentrations.

Highly reduced conditions occurred in Louisiana rice soils at the beginning of the flowering stage accompanied by a peak of H_2S accumulation at toxic levels during both heading-flowering and grain formation stages of rice plant development. Reduction intensity in rice soils has been reported (4, 14, 51, 56, 70) and used as a diagnostic criterion for the accumulation of reduction products (34). However, no direct measurements of these products, especially H_2S, have been made in previous studies. The nature, extent and biological role of sulfides in rice fields determined empirically in the present work indicate that previous findings have led to erroneous conclusions. For example, previous findings have failed to detect the consistent

occurrence of low concentrations of soluble sulfides in submerged soils (56).

Contrary to the agronomic view (34, 56) that ferrous iron plays a successful suppressive role against H_2S accumulation in rice soils, the present work has revealed that H_2S does indeed accumulate and that the three factors of greatest importance in H_2S accumulation are oxidizable carbon, soil pH, and manganous-manganese. Soil pH appeared to be the key factor in H_2S production and accounted for about 84.55 % of the accumulated H_2S at the heading-flowering stage.

It is evident that clay type (36) rather than clay percentage is a factor in sorption and removal from the soil solution of measurable amounts of H_2S. This sorption could be attributed to a combination of physical adsorption and chemical fixation of the sulfide ion. Absence of such sorption provides a reason for the occurrence of Straighthead disease of rice on sandy soils such as Hockley fine sandy loam (5, 24), for the type of clay in such soils does not sorb H_2S.

Clay sorption points up the limitations of chemical theory in predicting soluble sulfide levels under field conditions, and the empirical evidence reported here is in support of this view. The measured values of H_2S at the heading-flowering stage were significantly higher than predicted by chemical theory (24).

Inhibition of in vitro root respiration at all levels of H_2S confirmed the linear nature of this inhibition discovered by Mitsui (44, 45), and also extends the effects of H_2S to lower concentrations under controlled experimental conditions. These experimental conditions revealed that H_2S loss occurred from the culture solutions. Most of this loss probably resulted from volatilization into the atmosphere; other losses may have resulted from seedling uptake and carbonate formation.

H_2S levels toxic to rice plants in many studies reported in the literature (44, 45, 50, 55, 78) could lie below the range of 0.1-.07 ppm. Furthermore, the administered H_2S concentrations were within the range of concentrations detected under Louisiana rice field conditions with the sulfide electrode. It would be of great interest to know the effects of these low-inhibiting levels of H_2S on rice plants during the entire growth period under rice field conditions, as well as their in vitro effects under a regulated H_2S-flow control system.

The inhibition of oxidases is noteworthy. The physiological role of catalase and peroxidase is thought to be protective (48, 53, 54). In rice plant metabolism it is possible that peroxidase utilizes H_2O_2 produced by the glycolic acid pathway and/or as an intermediate of respiration. Peroxidases are responsible for lignin synthesis, for cell wall component synthesis, and for controlling the auxin contents of plant tissues (8). Also, catalase may function as a peroxidase at low H_2O_2 concentrations (8, 18, 48, 53).

The glycolic acid pathway has been demonstrated in rice root respiration (8, 41, 42, 43), and according to this theoretical scheme, which has some experimental support, the oxidative power of the rice root is primarily due to decomposition of H_2O_2 produced in this pathway. Futhermore, the prevention of formation and/or inhibition of catalase and peroxidase activity, demonstrated in the present work, may alter the physiological course of rice plant development at critical growth stages (7, 8, 30), and this could affect grain yield and cause "symptomless" (toxicant) diseases (24). Physiological disorders or diseases have been

connected with accumulation of H_2S in rice soils and reported in the literature for several rice growing areas of the world (72, 73).

At present there remains no doubt that cytochrome oxidase is the terminal oxidase in plants (7, 8, 29, 30, 46). Inada (30) and others (7, 8, 29, 46) demonstrated the active role of cytochrome oxidase in the young roots of rice plants and showed that it decreases with their aging, but a high activity is often observed in older roots. Cytochrome oxidase activity in this study was apparent in the enzyme extract. However, the slope of this activity was poorly defined and the activity was more apparent in younger roots.

Whatever the physiological role of the copper-containing enzymes may be, their inhibition by H_2S concentrations similar to H_2S levels in Louisiana rice fields could alter the physiological course of these enzymes and affect the normal growth of the rice plant. Thus, it appears that the H_2S inhibition of all four oxidases--catalase, peroxidase, ascorbic acid oxidase, and polyphenol oxidase--as well as cytochrome terminal oxidase--will affect the rice root oxidative capacity and physiological functions.

There is some possibility that oxidases (such as flavoproteins) which transfer reducing equivalents to oxygen, and the copper-containing enzymes (ascorbic acid oxidase and polyphenol oxidase) also function as terminal oxidases in association with respiratory chains, whether under normal and/or abnormal physiological conditions. However, such systems have not been actually observed (53). Inhibition of rice root respiration with KCN indicated the presence of flavin oxidase systems (7, 8, 18, 29, 46). These systems would prevent complete inhibition of

respiration by H_2S; residual respiration would always occur in H_2S exposed seedlings.

The results are highly suggestive that H_2S at low levels is possibly a yield-reducing factor in Gulf coast (USA) rice fields.

SUMMARY

I. The sulfide electrode studies showed that:

1. Evaluation of the silver-sulfide membrane electrode (sulfide electrode) has revealed sensitivity, selectivity, and other response characteristics well-suited to the analytical utilization of this electrode in field and laboratory studies.

2. Calibration curves obtained by using the sulfide electrode obeyed the Nernstian equation and were reproducible.

3. The sulfide electrode can be used to determine either the concentration of free sulfide ion or the total sulfide concentration if the pH value of the sample solution is determined simultaneously.

4. Also, sulfide ion concentrations can be determined by direct potentiometric titration in sample solution with great accuracy and reproducibility.

5. The response rate of the sulfide electrode versus the sample pH showed a uniform experimental slope of $30 \pm .5$ mv/pH unit in agreement with that predicted by the Nerstian equation.

6. The sulfide electrode responded in a reversible manner to the sample pH as well as to the $pS^{=}$ in sulfide electrolytes.

7. The methylene blue method gave results similar to those obtained with the sulfide electrode.

8. The correlation coefficient ($r = .9965$) between H_2S values determined by the methylene blue method and the sulfide electrode was highly significant ($P < 0.001$).

9. The sulfide electrode is practical and convenient for measuring free sulfide in situ.

II. H_2S levels in Louisiana rice soils showed that:

1. Significant measurable amounts of H_2S accumulated in soils under rice from the time of its tillering to its ripening. Sampling studies were confined to this period and the results are not meant to imply any information about sulfide levels at any other period during the growing season.

2. The peak of H_2S accumulation occurred at the heading-flowering stage and then began to decrease.

3. In some fields, H_2S levels were greater than or equal to reported values toxic to rice seedlings.

4. The overall mean for the tested 53 sites was .1037 ppm H_2S which exceeded toxic levels to rice plants in vitro.

5. Sixteen sites showed H_2S in amounts greater than 0.1 ppm.

6. Significant changes in pH and Eh_7 corresponded with those of H_2S accumulation.

7. The pH values showed a significant decrease with time varying from pH 6.9-6.3 at the booting stage to 6.5-5.4 at the heading-flowering stage to 6.3-5.4 at the ripening stage.

8. The average of Eh_7 varied from 4.7 at the booting stage and -50.25 at the heading-flowering stage to -37.0 at the ripening stage.

9. A peak of H_2S accumulation occurred during both heading-flowering and grain formation stages.

10. Measured H_2S values in rice fields during the heading-flowering stage were significantly higher than those predicted by chemical equilibrium theory.

11. The two most important factors in H_2S accumulation were oxidizable carbon and soil pH.

12. Production and accumulation of H_2S decreased by 0.39 units (ppm) per each one-unit increase in soil pH.

13. Ferrous iron concentration and soil Eh had no appreciable effect on H_2S accumulation.

14. The soil clay fraction, however, appeared to have a significant effect on H_2S accumulation.

15. Bentonite clay, a major constituent of some important rice soils, showed $S^{=}$ sorption in the range of 2.68-2.82 ppm/gm. This sorption could be divided between physical adsorption and chemical fixation.

16. It appears that the type of clay rather than the percentage of clay is important with respect to H_2S accumulation.

17. About 50% of H_2S at low concentrations (3.5-0.1 ppm) was lost linearly with time over a 6 hr period from water solutions.

III. <u>The rice seedling biological assay showed that</u>:

1. Rice root respiration was significantly inhibited by all administered levels of H_2S when compared to the control.

2. Percent inhibition of root respiration was a function of H_2S concentration and exposure period.

3. Hydrogen cyanide (CN)-resistant respiration systems (flavoprotein) were inferred from residual respiration in rice seedlings.

4. Catalase activity of rice root tissue was inhibited 30% and 24% at 2.4 and 1.2 ppm of H_2S, respectively after an initial 5 hr pretreatment in an H_2S solution.

5. Peroxidase activity of rice root tissue was inhibited 10.7 and 55.4 percent at 0.1 and 3.2 ppm of H_2S, respectively after an initial 6 hr pretreatment in an H_2S solution.

6. Ascorbic acid oxidase activity of rice root tissue was inhibited 29 and 40% at 0.1 and 3.2 ppm H_2S, respectively after an initial 6 hr pretreatment in an H_2S solution.

7. Polyphenol oxidase activity of rice root tissue was inhibited by 8 and 38% at 0.1 and 3.2 ppm H_2S, respectively after an initial 6 hr pretreatment in an H_2S solution.

LITERATURE CITED

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VITA

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LIST OF PUBLICATIONS

1. Allam, A. I. and J. B. Sinclair. 1968. Uptake and movement of Vitavax by germinating cottonseed. Phytopathology 58:1041. (Abstr.)

2. Allam, A. I. and J. B. Sinclair. 1969. Degradation of DMOC (Vitavax) in cotton seedlings. Phytopathology 59:1548-1549.

3. Allam, A. I., G. R. Pitts, and J. P. Hollis. 1971. Hydrogen sulfide levels in rice rhizosphere. Soil Science (Submitted for publication).

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EXAMINATION AND THESIS REPORT

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Major Field: Plant Pathology

Title of Thesis: SOLUBLE SULFIDES IN RICE FIELDS AND THEIR IN VITRO EFFECTS ON RICE SEEDLINGS

Approved:

John P. Hollis
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